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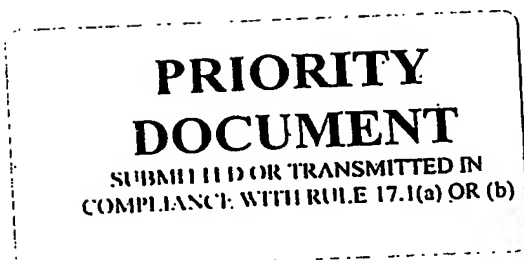
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Use of prion conversion modulating agents

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USE OF PRION CONVERSION MODULATING AGENTS

Field of the invention

5 This invention relates to the use of apolipoprotein B or apolipoprotein E or fragments thereof for diagnostic, detection, prognostic and therapeutic applications in prion diseases. More specifically, the invention provides the use of apolipoprotein B or apolipoprotein E or fragments thereof for modulating or identifying modulators of the prion protein replication which are implicated in the pathogenesis of transmissible spongiform encephalopathies and other prion diseases.

10

Background of the invention

Creutzfeldt-Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals are some of the diseases that belong to the group of Transmissible Spongiform Encephalopathies (TSE), also known as prion diseases
15 (*Prusiner, 1991*). These diseases are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease (*Roos et al., 1973*). To date no therapy is available.

Although these diseases are relatively rare in humans, the risk for the transmissibility of
20 BSE to humans through the food chain has seized the attention of the public health authorities and the scientific community (*Soto et al., 2001*). Variant CJD (vCJD) is a new disease, which was first described in March 1996 (*Will et al., 1996*). In contrast to typical cases of sporadic CJD (sCJD), this variant form affects young patients (average age 27 years old) and has a relatively long duration of illness (median 14 months vs. 4.5
25 months in traditional CJD). A link between vCJD and BSE was first hypothesized because of the association of these two TSEs in place and time (*Bruce, 2000*). The most recent and powerful evidence comes from studies showing that the transmission characteristics of BSE and vCJD to mice are almost identical and strongly indicating that they are due to the same causative agent (*Bruce et al., 1997*). Moreover, transgenic
30 mice carrying a human or a bovine gene have now been shown to be susceptible to BSE and vCJD (*Scott et al., 1999*). Furthermore, no other plausible hypothesis for the occurrence of vCJD has been proposed and intensive CJD surveillance in five European

countries, with a low exposure to the BSE agent, has failed to identify any additional cases. In conclusion, the most likely cause of vCJD is exposure to the BSE agent, probably due to dietary contamination with affected bovine central nervous system tissue.

5

The nature of the transmissible agent has been matter of passionate controversy. Further research, has indicated that the TSE agent differs significantly from viruses and other conventional agents in that it seems not to contain nucleic acids (*Prusiner, 1998*). Additionally, the physicochemical procedures that inactivate most viruses, such as
 10 disrupting nucleic acids, have proved ineffective in decreasing the infectivity of the TSE pathogen. In contrast, the procedures that degrade protein have been found to inactivate the pathogen (*Prusiner, 1991*). Accordingly, the theory that proposes that the transmissible agent is neither a virus nor other previously known infectious agent, but rather an unconventional agent consisting only of a protein recently gained widespread
 15 acceptability (*Prusiner, 1998*). This new class of pathogen was called a "prion", short for "proteinaceous infectious particle". In TSE, prions are composed mainly of a misfolded protein named PrP^{Sc} (for scrapie PrP), which is a post-translationally modified version of a normal protein, termed PrP^C (*Cohen et al., 1998*). Chemical differences have not been detected to distinguish these two PrP isoforms and the
 20 conversion seems to involve a conformational change whereby the α -helical content of the normal protein diminishes and the amount of β -sheet increases (*Pan et al., 1993*). The structural changes are followed by alterations in the biochemical properties: PrP^C is soluble in non-denaturing detergents, PrP^{Sc} is insoluble; PrP^C is readily digested by proteases (also called protease sensitive prion protein) while PrP^{Sc} is partially resistant,
 25 resulting in the formation of a N-terminally truncated fragment known as PrPres (protease resistant prion protein) (*Cohen et al., 1998*).

The notion that endogenous PrP^C is involved in the development of infection is supported by experiments in which endogenous PrP gene was knocked out where the animals were both resistant to prion disease and unable to generate new infectious
 30 particles (*Bueler et al., 1993*). In addition, it is clear that during the time between the inoculation with the infectious protein and the appearance of the clinical symptoms, there is a dramatic increase in the amount of PrP^{Sc}.

These findings suggest that endogenous PrP^C is converted to PrP^{Sc} conformation by the action of an infectious form of the PrP molecule (*Soto et al., 2001*). Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^C, catalyzing its conversion to the pathogenic form of the protein. A physical association between the two isoforms during the infectious process is suggested by the primary sequence specificity in prion transmission (*Telling et al., 1994*) and by the reported *in vitro* generation of PrP^{Sc}-like molecules by mixing purified PrP^C with PrP^{Sc} (*Saborio et al., 2001*). However, the exact mechanism underlying the conversion is not known.

Investigations with chimeric transgenes showed that PrP^C and PrP^{Sc} are likely to interact within a central domain delimited by codons 96 and 169 (*Prusiner, 1996*) and synthetic PrP peptides spanning the region 109-141 proved to be able to bind to PrP^C and compete with PrP^{Sc} interaction (*Chabry et al., 1998*).

Based on data with transgenic animals, it has been proposed that additional brain factors present in the host are essential for prion propagation (*Telling et al., 1995*). It has been demonstrated previously that prion conversion does not occur under experimental conditions where purified PrP^C and PrP^{Sc} are mixed and incubated (*Saborio et al., 1999*) but that the conversion activity is recovered when the bulk of cellular proteins are added back to the sample (*Saborio et al., 1999*). This finding provides direct evidence that other factors present in the brain are essential to catalyze prion propagation.

The observation that cholesterol depletion decreases the formation of PrP^{Sc} whereas sphingolipid depletion increases PrP^{Sc} formation, suggested that “lipid rafts” (lipid domains in membranes that contain sphingolipids and cholesterol) may be the site of the PrP^C to PrP^{Sc} conversion reaction involving either a raft-associated protein or selected raft lipids (*Fantini et al., 2002*). However, the role of lipid rafts in prion infectivity is still unclear.

One approach to the treatment and prevention of prion diseases has been to develop agents for blocking the transformation of PrP^C into PrP^{Sc}. Some agents proposed were Congo red dye (US 5,276,059), nerve growth peptides (US 5,134,121), fragments of

prion proteins (US 6,355,610) and beta-sheet breaker peptides (US 5,948,763) but it would be desirable to develop new methods for identifying and inhibiting the prion conversion factor(s).

5 Apolipoprotein B is the major protein component of the two known atherogenic lipoproteins, Low Density Lipoproteins (LDL) and remnants of triglyceride-rich lipoproteins and is a ligand for the LDL receptor (*Segrest et al., 2001*). Apolipoprotein E is a constituent of several plasma lipoprotein such as chylomicrons, very low-density lipoproteins (VLDL), and high-density lipoproteins (HDL) (*Lehninger et al., 1993*) and
10 is a ligand for the LDL receptor. Apolipoprotein B and E are known for their prominent role in cholesterol transport and plasma lipoprotein metabolism via LDL receptor interactions. Apolipoprotein E has recently emerged as a major genetic risk factor for Alzheimer's disease, a neurodegenerative disorder.

15 **Summary of the invention**

It is an object of the invention to provide a use of peptides or proteins in an assay for the detection of PrP^{Sc} formation in a sample.

It is also an object of the invention to provide a use of peptides or proteins in a
20 screening assay for identifying compounds that modulate the conversion of PrP^c into PrP^{Sc}.

It is further an object of the invention to provide a substance which is suitable for the treatment of and/or prevention of and/or delaying the progression of prion related
25 disorders, notably, bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD).

In a first aspect, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and
30 mimetics thereof in an assay for the detection of PrP^{Sc} formation in a sample.

In a second aspect, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and

mimetics thereof in a screening assay for identifying compounds that modulate the conversion of PrP^C into PrP^{Sc}.

5 In a third aspect, the invention provides a use of a modulator, preferably an inhibitor or an antagonist, of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof for the preparation of a pharmaceutical preparation for the treatment of a prion disease, notably, bovine spongiform encephalopathy (BSE) and a Creutzfeld-Jacob Disease (CJD).

10 In a fourth aspect, the invention provides a method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) obtaining a sample from the subject; (ii) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (iii) contacting the sample
15 obtained from step (ii) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample.

In a fifth aspect, the invention provides a method of determining a marker that
20 predisposes a subject to a prion disease, comprising (i) obtaining a sample from the subject; (ii) measuring a level of a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment and mimetics thereof in said sample; and (iii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease.

25

In a sixth aspect, the invention provides a method for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (ii) contacting the sample obtained from step (i)
30 with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a seventh aspect, the invention provides a method for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound.

In a eighth aspect, the invention provides an assay for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a ninth aspect, the invention provides a screening assay for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound.

Detailed description of the invention

The following paragraphs provide definitions of various terms, and are intended to apply uniformly throughout the specification and claims unless an otherwise expressly set out definition provides a different definition.

5

The term "Gerstmann-Strassler-Scheinker Disease" abbreviated as "GSS" refers to a form of inherited human prion disease. The disease occurs from an autosomal dominant disorder. Family members who inherit the mutant gene succumb to GSS.

10 The term "prion" shall mean a transmissible particle known to cause a group of such transmissible conformational diseases (spongiform encephalopathies) in humans and animals. The term "prion" is a contraction of the words "protein" and "infection" and the particles are comprised largely if not exclusively of PrP^{Sc} molecules.

15 "Prions" are distinct from bacteria, viruses and viroids. Known prions include those which infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats as well as bovine spongiform encephalopathies (BSE) or mad cow disease and feline spongiform encephalopathies of cats. Four prion diseases known to affect humans are Kuru, Creutzfeldt-Jakob Disease (CJD),
 20 Gerstmann-Strassler-Scheinker Disease (GSS), and fatal familial insomnia (FFI) (*Prusiner, 1991*). As used herein prion includes all forms of prions causing all or any of these diseases or others in any animals used -- and in particular in humans and in domestic farm animals.

25 The term "lipid rafts" refers to small platforms, composed of sphingolipids and cholesterol in the outer exoplasmic layer, connected to Cholesterol in the inner cytoplasmic layer of the bilayer that have been reviewed recently (*Simons et al., 2000*). Lipid rafts can be isolated as they are insoluble in certain detergents such as triton X-100 at 4°C. Therefore, rafts can be purified as detergent-insoluble membranes (DIMs)
 30 or detergent-resistant membranes (DRMs) by ultracentrifugation on sucrose gradients. Rafts are enriched in GPI-anchored proteins, as well as proteins involved in signal transduction and intracellular trafficking. In neurons, lipid rafts act as platforms for the

signal transduction initiated by several classes of neurotrophic factors (*Tsui-Pierchala et al., 2002*). Example for lipid rafts extraction is given in Example n° 2 §c.

5 The term “prion conversion factor” refers to a factor comprising proteins, lipids, enzymes or receptors that acts as a co-factor or auxiliary factor involved in the process of conversion of PrP^C into PrP^{Sc} and favors the onset and/or progression of the prion disease.

10 The terms “standardized prion preparation”, “prion preparation” and the like are used interchangeably herein to describe a composition containing prions which composition is obtained for example from brain tissue of mammals substantially the same genetic material as relates to PrP proteins, e.g. brain tissue from a set of mammals which exhibit signs or prion disease or for example a composition which is obtained from chronically prion infected cells.

15 The terms “sensitive to infection”, “sensitive to prion infection” and the like are use for a material from a mammal, including cells, that can be infected with an amount and type of prion which would be expected to cause prion disease or symptoms.

20 By analogy, the terms “resistant to infection”, “resistant to prion infection” and the like are used for a material from a mammal, including cells which has the characteristic to be resistant when infected with an amount and type of prion which would be expected to cause prion disease or symptoms and remain uninfected even after several infective prion material inoculations.

25 The term “sample” refers to a biological extract from a mammal, including cell sample, body fluid, genetic material such as brain homogenate, cells, lipid rafts or purified peptides and proteins.

30 The term “incubation time” shall mean the time from inoculation of an animal with a prion until the time when the animal first develops detectable symptoms of disease resulting from infection, it also means the time from inoculation of material from a

mammal, e.g. brain homogenate, cells, lipid rafts from cells, with prion until the time when the prion infection is detectable such as through the conversion of PrP^C into PrP^{Sc}. Several methods of detection of prion infection and PrP conversion are known by a person skilled in the art.

5

The term "fraction" refers to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the original polypeptide or peptide. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps.

15

The terms "modulator" or "modulatory compound" refer to molecules that modify the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, metabolism) of the natural protein. "Modulators" of "modulatory compounds" include "agonists" and antagonists". Modulators" include peptides, proteins or fragments thereof, peptidomimetics, organic compounds and antibodies.

20

The term "mimetics" refer to molecules that mimic the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, metabolism) of a natural protein. These compounds have for example the property to either enhance a property of the natural protein (i.e. to lead to the same activity when the compound is added to the natural protein as obtained with an increase in concentration in the natural protein) or to exhibit the same property as a natural protein (i.e. to lead to the same activity when the compound replaces the natural protein). "Mimetics" include peptides, proteins or fragments thereof, peptidomimetics and

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organic compounds. Examples of apolipoprotein E mimetics are described in US20020128175.

5 The terms "inhibitor" or "antagonist" refer to molecules that alter partially or impair the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, secretion, metabolism) of the natural protein. "Inhibitors" or "antagonists" include peptides, proteins or fragments thereof, peptidomimetics, organic compounds and antibodies. Examples of Apolipoprotein B antibodies are described in *Choi et al., 1997* and in *Wang et al., 2000*. Examples of 10 Apolipoprotein E antibodies are described in *Aizawa et al., 1997* and *Yamada et al., 1997*. Examples of Apolipoprotein antagonists can be antagonists that alter or impair the role of Apolipoproteins B or E in the cholesterol transport pathway. Examples of compounds that alter Apolipoprotein B secretion or synthesis are described in US 6,369,075, US 6,197,972, WO 03002533 and WO 03045921. Other "modulators" or 15 "antagonists" can be modulators of the LDL receptor, preferably LDL-receptor antagonists such as anti-LDL receptor antibodies. Examples of monoclonal antibodies to the LDL receptor are given in WO 0168710.

20 The term "protein misfolding cyclic amplification assay" or "PMCA assay" is an assay that for the diagnosis or detection of conformational diseases which comprises a cyclic amplification system to increase the levels of the pathogenic conformer such as described for example in WO 0204954.

25 The term "marker" for a disease refers to a biological parameter or value including a genetic character, inherited protein mutation(s), blood level of a protein or an enzyme that is different from the average value in a heterogeneous population of individuals and whose occurrence correlates with the occurrence of said disease with a statistical significance. A "marker" for a disease or condition is typically defined as a certain cut-off level of a said biological variable. A "marker" provides basis for determining the 30 risk (probability of occurrence) of a disease in a subject.

The term "complex" includes the formation of an entity by the interaction of several molecules, several proteins, several peptides together or with a receptor. These interactions may be reversible and/or transient. These interactions may induce changes in the properties of the interacting molecules, proteins, peptides or receptors.

5

By "effective amount", it is meant a concentration of peptide(s) that is capable of slowing down or inhibiting the formation of PrP^{Sc} deposits, or of dissolving preformed deposits. Such concentrations can be routinely determined by those of skill in the art. It will also be appreciated by those of skill in the art that the dosage may be dependent on the stability of the administered peptide. A less stable peptide may require administration in multiple doses.

10

The preparation of antibodies is known by the person skilled in the art. It is referred by "antibody" to a monoclonal antibody, chimeric antibody, humanized antibody, anti-anti-
15 Id antibody or fragment thereof which specifically recognises and binds to Apo B or Apo E and fragments thereof. For example, monoclonal antibodies are obtained through the generation of hybridoma cells lines producing monoclonal antibodies capable of specifically recognising and binding Apo B or Apo E and fragments thereof. More specifically, these monoclonal antibodies are capable of specifically recognising and
20 binding Apo B or Apo E. A monoclonal antibody can be prepared in a conventional manner, e.g. by growing a cloned hybridoma comprising a spleen cell from a mammal immunized with hApo B or hApo E and a homogenic or heterogenic lymphoid cell in liquid medium or mammalian abdomen to allow the hybridoma to produce and accumulate the monoclonal antibody. Preferably, the antibody specifically recognises
25 and binds to Apo B- and/or Apo E-LDL recognizing fragments.

20

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The present invention provides compounds capable of controlling, including increasing and/or inhibiting, the conversion of PrP^C into PrP^{Sc} in prion diseases.

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The activity of the compounds of the invention in controlling the conversion of PrP^C into PrP^{Sc} in prion diseases can be detected using, for example, an *in vitro* assay, such as that described by Saborio *et al.*, 2001 which measures the ability of compounds of the

invention to modulate the conversion of PrP^C into PrP^{Sc}. Results are reported in the Examples.

5 In an embodiment of the invention, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof in an assay for the detection of PrP^{Sc} formation in a sample.

10 In one preferred embodiment of the invention, the peptide or the protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof used in an assay for the detection of PrP^{Sc} formation in a sample binds and/or forms a complex with the LDL receptor.

15 In another embodiment, the invention provides a use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof in a screening assay for the identifying compounds that modulate the conversion of PrP^C into PrP^{Sc}.

20 In another preferred embodiment of the invention, the peptide or the protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof used in a screening assay for the identifying compounds that modulate the conversion of PrP^C into PrP^{Sc} binds and/or forms a complex with the LDL receptor.

25 In a further embodiment of the invention, the assay is a Protein Misfolding Cyclic (PMCA) assay.

In a preferred embodiment of the invention, the Protein Misfolding Cyclic (PMCA) assay uses normal brain homogenate as a source of normal PrP^C and prion conversion factor.

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In a preferred embodiment of the invention, the Protein Misfolding Cyclic (PMCA) assay uses cell lysates or lipid rafts extracted from prion infection sensitive neuroblasma

cells, such as line N2a, described in Example 2, and equivalent, as a source of normal PrP^c and prion conversion factor. Lipid raft fractions can also be purified directly from the brain to serve as a source of substrate for PMCA.

- 5 In another embodiment, the invention provides a use of a modulator, preferably an inhibitor or an antagonist, of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof for the preparation of a pharmaceutical preparation for the treatment of a prion disease, notably, bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD). The modulator
10 modifies for example the functions and/or properties of Apo B and/or Apo E or of a fragment thereof.

- In a preferred embodiment of the invention, the modulator, preferably an inhibitor or an antagonist, of a peptide or a protein selected from Apolipoprotein B, a fragment thereof,
15 Apolipoprotein E and a fragment thereof modifies, preferably inhibits the binding and/or the formation of a complex between Apolipoprotein B and/or Apolipoprotein E and the LDL receptor. An example of such modulator can be a LDL receptor modulator, a preferably LDL-receptor antagonist such as an anti-LDL receptor antibody.

- 20 In a preferred embodiment of the invention, the modulator is an antibody raised against Apolipoprotein B or against a fragment thereof.

- In another preferred embodiment of the invention, the modulator is an antibody raised against Apolipoprotein B.
25

- In another preferred embodiment of the invention, the modulator is an antibody raised against a fragment of Apolipoprotein B, which fragment is of, or about, a molecular weight selected from 30, 35 and 40 kDa.

- 30 In another preferred embodiment of the invention, the modulator is an antibody raised against a fragment of Apolipoprotein B, which fragment comprises a sequence selected

from fragments taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

5 In a preferred embodiment of the invention, the peptide or protein is selected from Apolipoprotein B or a fragment thereof.

In another preferred embodiment of the invention, the peptide or protein is selected from Apolipoprotein E or a fragment thereof.

10 In a preferred embodiment of the invention, the peptide or protein contains the sequence of SEQ ID NO: 3.

In another preferred embodiment of the invention, the peptide or protein is a fragment which is of, or about, a molecular weight selected from 30, 35 and 40 kDa.

15

In another preferred embodiment of the invention, the peptide or protein is a fragment of Apolipoprotein B, comprising a sequence selected from fragments, taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

20 In an embodiment of the invention, the invention provides a method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) obtaining a sample from the subject; (ii) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (iii) contacting the
25 sample obtained from step (ii) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample from the subject includes a biological extract from a mammal such as cell sample, genetic material, body fluid, brain homogenate, cells and lipid rafts.

30

In another embodiment of the invention, the invention provides a method of determining a marker that predisposes a subject to a prion disease, comprising (i)

- obtaining a sample from the subject; (ii) measuring a level of a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment and mimetics thereof in said sample; (iii) contacting the sample obtained from step (ii) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease. The maker includes a biological parameter or value such as a genetic character, inherited protein mutation(s), blood level of a protein or an enzyme.
- 10 In another embodiment of the invention, the invention provides a method for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (iii) contacting the sample obtained from step (ii) with PrP^C or PrP^C containing mixtures, such as brain
- 15 homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample can be a biological preparation for which the presence of prion is to be detected for quality control reasons and/or a sample extracted from a subject that is suspected of suffering of such a disease, including a biological extract from a mammal such as cell sample, genetic material, body fluid,
- 20 brain homogenate, cells and lipid rafts.

In another embodiment of the invention, the invention provides a method for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected

25 from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said compound; (iii) contacting the sample obtained from step (i) a and (i) b, with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the amount of PrP^{Sc} (a) in the presence of said

30 compound and (b) in the absence of said compound. The modulator, includes antibodies, inhibitors of Apolipoproteins B and/or E binding, including binding to the LDL receptor, and/or secretion and/or synthesis.

Still another embodiment of the present invention, is a method for treating or preventing a prion disease such as bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD), wherein the method comprises administering an effective dose of the
5 above-mentioned modulator of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof, to a subject in the need thereof, wherein the subject can be human or animal.

10 In a preferred method of use of the modulators, preferably inhibitors, administration of the modulators is by injection or infusion, at periodic intervals. The administration of a compound of the invention may begin before any symptoms are detected in the patient, and should continue thereafter.

The above-mentioned modulatory compounds of the present invention may
15 be administered by any means that achieves the intended purpose. For example, administration may be by a number of different routes including, but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intra-cerebral, intrathecal, intranasal, oral, rectal, transdermal, intranasal or buccal. Preferably the compounds of the invention are administered by subcutaneous, intramuscular or
20 intravenous injection or infusion.

Parenteral administration can be by bolus injection or by gradual perfusion over time. A typical regimen for preventing, suppressing, or treating prion related disorders, comprises either (1) administration of an effective amount in one or two doses of a high
25 concentration of modulatory in the range of 0.5 to 10 mg of peptide, more preferably 0.5 to 10 mg of peptide, or (2) administration of an effective amount of the peptide in multiple doses of lower concentrations of modulatory compounds in the range of 10-1000 µg, more preferably 50-500 µg over a period of time up to and including several months to several years. It is understood that the dosage administered will be dependent
30 upon the age, sex, health, and weight of the recipient, concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients which are known in the art. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspension of the active compound as appropriate oily injections suspensions may be administered.

In another embodiment of the invention is provided an assay for the detection of the formation of PrP^{Sc} within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof; (iii) contacting the sample obtained from step (ii) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample can be a biological preparation for which the presence of prion is to be detected for quality control reasons and/or a sample extracted from a subject that is suspected of suffering of such a disease, including a biological extract from a mammal such as cell sample, genetic material, body fluid, including blood, serum, plasma, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, is provided a screening assay for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E, a fragment thereof and mimetics thereof (a) in the presence of said compound and (b) in the absence of said modulatory compound; (iii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said modulatory compound. The modulator, includes antibodies, inhibitors of Apolipoproteins B and/or E binding and/or secretion and/or synthesis.

In further embodiment of the invention, is provided a diagnostic kit for use in the assay of the invention, comprising a probe for receiving a sample and a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof. The kit of the invention comprises kits having multi-well microtitre plate and/or multi-well sonicator.

In a still further embodiment of the invention, is provided an apparatus for use in the methods of the invention or in the assays of the invention. The apparatus of the invention comprises apparatus that have a microtitre plate and/or multi-well sonicator.

In a preferred embodiment, the prion disease is bovine spongiform encephalopathy (BSE).

In a preferred embodiment, the prion disease is sporadic, variant, familial or iatrogenic Creutzfeld-Jacob Disease (CJD).

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

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Brief description of the drawings:

Figure 1 shows *in vitro* prion replication on Hamster brain homogenate by PMCA assay in presence and absence of a cholesterol-depleting agent (Example 1§b). Samples contain 5% normal hamster brain homogenate incubated for 30 min at 4°C with 0, 5, 10 or 20 mM (final concentration) of methyl- β -cyclodextrin (M β CD). Aliquots of scrapie brain homogenate are added to reach a 3200- (top panel) and 12800-fold (bottom panel) dilution. Half of the samples are frozen immediately as a control

without amplification (PMCA “-“) and the other half are subjected to 10 cycles of PMCA (PMCA “+“). Prion replication is evaluated by Western Blot after treatment of the samples with PK (100µg/ml for 60 min). The first lanes in each blot corresponds to the normal brain homogenate not treated with PK.

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Figure 2 shows the effect of Apolipoproteins B, E and J *in vitro* prion replication on Hamster brain homogenate by PMCA assay (Example 1§c). Samples containing 5% normal hamster brain homogenate are incubated with different quantities of human ApoB (2A), human ApoE (2B) or murine ApoJ (2C) for 30 min at 4°C. Aliquots of scrapie brain homogenate are added to reach a 3200- (left panel) or 12800-fold (right panel) dilution. Half of the samples are frozen immediately as a control without amplification (PMCA “-“) and the other half are subjected to 10 cycles of PMCA (PMCA “+“). Prion replication is evaluated by western blot after treatment of the samples with PK (100µg/ml for 60 min). The first lanes in each blot corresponds to the normal brain homogenate not treated with PK.

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Figure 3 reports differential sensitivity of N2a sub-clones to infection by Scrapie revealed by exposure to anti-PrP 6H4 mabs (Example n° 2 §b). Proteinase K (PK) exposure shows were the PrP^{Sc} isoform (Proteinase K resistant) is present. The two sub-clones highlighted #23 and #60 are chosen respectively as representatives of prion infection resistant and sensitive cells. 'N2a' shows uninfected N2a cells processed in parallel. Controls for blotting and PK digestion show 1µl normal or scrapie brain extract diluted in 80µl lysis buffer and processed in parallel.

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Figure 4 shows the characterization of PrP in lipid rafts from sub-clones prion infection resistant (#23) and sensitive (#60) N2a (Example 2 §c). Figure 4A shows PrP quantification by Western blotting in lipid rafts which are extracted from prion infection resistant (#23) and sensitive (#60) cells. The distribution of PrP in the total extract (25µg loaded) (1), the sucrose sample layer after centrifugation (25µg loaded) (2) and the bouyant lipid raft fraction (4µg loaded) (3) are presented. Figure 4B shows PrP content and glycosylation pattern of the two sub-clones #23 and #60 by Western

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blotting with anti-PrP. Three independent preparations of lipid rafts prion infection resistant (#23) and sensitive (#60) cells were analysed. Equal amounts (4µg) of rafts proteins were analysed in each case. Figure 4C shows the same membrane after stripping and re-probing with anti-actin which confirms the similarity in protein loading.

Figure 5 presents the *in vitro* conversion activity of lipid rafts from sub-clones prion infection resistant (#23) and sensitive (#60) N2a using PMCA (Example 2 §d). *Upper panel:* Lipid rafts are isolated from prion infection resistant (#23) and sensitive (#60) cells. Preparations are mixed in a ratio 100:1 with 10% RML brain homogenate and aliquots are frozen immediately, incubated for 15h at 37 °C or subjected to 15 cycles of PMCA. Lanes 1: initial mixture without PK digestion; lanes 2: initial mixture digested 10ug/ml PK 1hr 37 °C; lanes 3: mixture incubated 37 °C PK digested as in lane 2; lanes 4: 15 cycles of PMCA followed by PK digestion as in lane 2. Lane 5 shows the migration and cross-reactivity with anti-PrP of PK alone. *Lower panel:* Following western blotting the membrane is stained with Coomassie blue to confirm that digestion with PK was complete.

Figure 6 shows the inhibitory effect on Prion replication in prion infection sensitive cells induced by Anti-hApoB polyclonal antibody (Example 2 §e).

Chronically infected #60 sensitive cells were cultured in 24 well culture dishes in the presence of increasing amounts (0-2mg/ml) of a goat polyclonal antibody against human ApoB (Chemicon) or against a corresponding series of naïve goat IgG. The level of PrP replication was determined by quantitative dot blotting and expressed as chemiluminescent intensity/mg protein. In the graph, for each antibody concentration the chemiluminescent intensity is expressed as a percentage of the value obtained without the antibody. Higher concentrations of anti-hApoB antibody have an inhibitory effect on PrP replication.

Figure 7 shows 2D separations of lipid raft proteins from N2a cells (Example 3). Lipid rafts are isolated from prion infection sensitive cells (#60) and 2 aliquots of 25 µg are precipitated with acetone and processed for 2D analysis min the 1st dimension spanning pH ranges 3-10 (7A) or 6-11 (7B). After SDS-PAGE separation in the second

dimension, gels are stained using the silver express kit (Invitrogen). Arrow indicates the same protein on both gels (7A and 7B). Proteins within the rectangle shown in B are compared between lipid raft from the prion infection sensitive sub-clone #60, (C) and resistant subclone #23, (D). Arrows indicate proteins which are more abundant in resistant cells.

Abbreviations:

Apo B (Apolipoprotein B); Apo E (apolipoprotein E); Apo J (Apolipoprotein J); BCA (Bicinchoninic Acid); CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate); CNS (central nervous system); BSE (bovine spongiform encephalopathy); CJD (Creutzfeldt-Jakob Disease); DIM (Detergent-Insoluble Membrane); DRM (Detergent-Resistant Membrane); DTT (1,4-Dithio-D,L-threitol); IPG (Immobilized PH Gradient); IEF (Isoelectric Focusing); FFI (Fatal Familial Insomnia); GSS (Gerstmann-Strassler-Scheinker Disease); hr (hour); HRP (Horseradish Peroxidase); kDa (KiloDalton); LDL (Low Density Lipoprotein); μ g (microgram); μ l (microliter); min (minute); M β CD (methyl- β -cyclodextrin); mM (millimolar); MS (mass spectrometry); PBS (Phosphate Buffered Sulfate); PK (proteinase K); PMCA (Protein Misfolding Cyclic Amplification); PMSF (Phenylmethanesulfonyl Fluoride); PrP (prion protein); PrP^C (normal, non-pathogenic conformer of PrP); PrP^{Sc} (pathogenic or "scrapie" isoform of PrP which is also the marker for prion diseases); PVDF (polyvinylidene difluoride); RPM (Rotation per minute); RML (Rocky Mountain Laboratory); RT-PCR (reverse transcriptase polymerase chain reaction); SDS (Sodium Dodecyl Sulfate); V (Volt); Vol. (volume).

EXAMPLES

The invention will be illustrated by means of the following examples which are not to be construed as limiting the scope of the invention.

The following examples illustrate preferred compounds and methods for determining their biological activities.

PrP scrapie used as infection inoculum is RML (Rocky Mountain Laboratory) strain. Anti-PrP 6H4 monoclonal antibodies were purchased from Prionics.

Proteinase K was obtained from Boehringer Ingelheim and Methyl.β.cyclodextrin from Sigma.

Purified and delipidated human Apolipoprotein B and Apolipoprotein E were obtained from Chemicon.

5 Human Anti-apo B antibodies were obtained from Chemicon.

Mouse neuroblastoma N2a cell line was obtained from ATCC.

Murine Apo J was obtained in-house.

EXAMPLE 1: *In vitro* prion replication in brain homogenate through PMCA assay:

10 The influence of cholesterol and some of the apolipoproteins on prion replication in vitro is analysed through a Protein Misfolding Cyclic Amplification assay (PMCA) (*Saborio et al., 2001*) where hamster brain homogenate is used as a source of PrP^C and conversion factors as follows.

a) Brain preparation:

15 Brains from healthy Syrian golden hamsters healthy or infected with the adapted scrapie strain 263 K are obtained after decapitation and immediately frozen in dry ice and kept at -80° until used. Brains are homogenized in PBS containing protease inhibitors (Complete™ cocktail from Boehringer Mannheim) at a 1x final concentration. Detergents (0.5% Triton X-100, 0.05% SDS, final concentrations) are added and samples clarified with low speed centrifugation (10000 g) for 1 min, using an Eppendorf centrifuge (model 5415).

Dilutions (3200-fold and 12800-fold) of the scrapie brain homogenate are added directly to the healthy brain homogenate to trigger prion replication. 60μl of these mixtures are frozen immediately and another 60μl are incubated at 37°C with agitation. 25 Each hour a cycle of sonication (5 pulses of 1sec each) is done using a microsonicator (Bandelin Electronic, model Sonopuls) with the probe immersed in the sample and the power setting fixed at 40%. These cycles are repeated 10 times.

b) PMCA signal in presence and absence of a cholesterol-depleting agent:

30 Under these conditions a dramatic increase in the amount of PrP^{Sc} signal is observed after 10 cycles of PMCA (Figure 1, lanes 2 and 3). When normal brain

homogenate is treated during 30 min with 10 and 20mM (but not 5 mM) methyl- β -cyclodextrin (M β CD) a complete inhibition of prion replication is observed (Figure 1, lanes 6-9) as obtained in mouse models in cell cultures and in vitro, indicating that cholesterol depletion has a detrimental effect on prion replication (*Taraboulos et al.*,
5 1995).

c) PMCA signal in presence of apolipoproteins

Purified delipidated human ApoB (Figure 2A) and human Apo E (Figure 2B) are respectively added to the PMCA preparation without cyclodextrin at different
10 concentrations (8 and 16 μ g for hApo B) and (1 and 10 μ g for hApo E). Samples are incubated for 15 min at 4°C and thereafter half of each sample is frozen and the other half subjected to PMCA cycles.

An increase in prion replication in vitro is observed at both 3200-fold and 12800-fold dilutions of scrapie brain homogenate for both Apo B and Apo E.
15 In contrast addition of Apolipoprotein J (at concentrations of 1, 2 and 4 μ g), an Apolipoprotein component of HDL, has no effect on PMCA signal (Figure 2C).

These data show the effect of Apolipoproteins implicated B and E on prion conversion.

20 EXAMPLE 2: In vitro prion replication in lipid rafts from prion infection sensitive cells by PMCA:

The mouse neuroblastoma cell line N2a is used for their capability to be infected with PrP^{Sc}. *Baron et al.*, 2002 and *Enari et al.*, 2001 have shown that prion infection sensitive and prion infection resistant N2a sublines exist. Lipid rafts from the prion
25 infection sensitive line are isolated and used as a substrate for PMCA assay. The effect of an apolipoprotein B antagonist on prion conversion is studied through the ability of apolipoprotein B antagonist to inhibit the prion replication ability of prion infection sensitive N2a cell lines.

30 a) N2a cell preparation:

Sub-clones of the parental mouse neuroblastoma N2a cell line are derived from single cells by limit dilution. A growing culture (Dulbecco's Modified Eagles Medium

(DMEM Gibco # 41966-029), containing 10% fetal calf serum (FCS) and supplemented with 2mM, L-glutamate and standard antibiotics (penicillin and streptomycin)) is diluted to a density of 5 cells/ml and 100µl is transferred to individual wells of a 96 well plate and cultured for 1 week.

- 5 The individual cultures are examined microscopically to determine those wells which contained a single focus of growing cells. The single cell derived cultures are then transferred to 24 well plates and serially passaged every 3-4 days at 1:15 dilution to maintain stocks. A total of 63 cultures are isolated and all tested for sensitivity to infection by the RML strain of PrP^{Sc}. To do this, 4µl of a 10% late stage infected brain
10 extract is added per well of newly passaged cells, and the cultures are left for a further 4 days to reach 100 % confluence. Cells were serially passaged thereafter in the absence of PrP^{Sc}. Tests showed that all trace of the initial inoculum disappeared by passage 4.

At this and later passages individual cultures are tested for the presence of PrP^{Sc}.

15 **b) Prion infection resistant cell isolation by Cell culture dot blotting:**

The presence of PrP^{Sc} in the 63 individual cell cultures is tested by cell culture dot blotting procedure in which lysis and proteinase K (PK) digestion are carried out directly in the culture dish. PK resistant PrP^{Sc} is detected by dot blotting to PVDF membranes and exposure to anti-PrP antibody as follows:

- 20 Infected cells are grown for 3-4 days in 24-well plates and washed once with PBS. 40µl DNaseI (1000U/ml in H₂O) is added to each well at room temperature for 5min, followed by 40µl proteinase K solution (20µg/ml in 100mMTris/HCl pH 7.4, 300mM NaCl, 1% Triton-X100, 1% sodium deoxycholate). Plates are incubated at 37°C for 1hr with gentle agitation. Proteinase K digestion is stopped by addition of 2µl of a
25 solution containing 80µg/ml PMSF, 10mMTris-HCl pH 8.0 and 1mg/ml bromophenol blue. 20µl aliquots are spotted onto PVDF membranes equilibrated with a degasses solution containing 192mM Glycine, 25mM Tris, 20% methanol. Membranes are then transferred to 3M guanidine Thiocyanate, 10mM Tris HCL pH 8.0 for 10min to denature proteins, rinsed in water and processed as for Western blotting using anti-PrP
30 6H4 (Prionics). Non-specific binding is blocked by incubation with 5% milk dissolved in PBS for 1hr. The membrane is then exposed to specific primary antibody anti-PrP 6H4, followed by HRP-conjugated secondary antibody each diluted as appropriate in

PBS, 0.3% Tween 20. Western blots are developed by ECLTM (Amersham) as directed according to the provider instructions.

The chemiluminescence signal from membranes is then analyzed directly using the Kodak Imagestation 440CF. The luminescence signal in each condition was
5 normalized for possible differences in cell growth. Total protein content of a parallel lysate untreated with proteinase K is determined using the BCA assay (Biorad) and results are expressed as intensity/ μ g protein.

Of the 63 sub-clones analysed, 9 were found to be capable of replicating PrP^{Sc}, albeit with differing efficiencies (Figure 3). The remaining 54 sub-clones were resistant
10 to infection. The most highly prion infection sensitive cell lines were selected for further study together with several prion infection resistant sub-clones with similar morphologies and doubling times. We have selected two of these cell lines: #23, a prion infection resistant clone, and #60 a prion infection sensitive clone.

These two cell lines have been maintained in culture for over 1 year and have
15 been infected with RML in many different occasions throughout this period: on every occasion sub-clone #60 was highly infectable whereas sub-clone #23 was totally resistant. Prion infection sensitive sub-clones could be maintained as a chronically infected cell culture by serial passaging at 1:15 or 1:20 dilution every 3 or 4 days respectively.

20 No evident morphological differences by microscopy were observed between the resistant or sensitive cells or between non-infected and infected cells.

To validate the clinical relevance of this cellular model of PrP replication, extracts of chronically infected N2a cells, or buffer alone, were injected into the hippocampus of normal mice by stereotactic injection. Injection of N2a extracts resulted
25 in onset of clinical symptoms of scrapie after 140 days and premature death whereas mock injection had no effect on mouse physiology or life span. This indicates that the cell based model for prion replication using prion infection sensitive N2a cells generates infectious PrP scrapie, confirming that the conversion of PrP in cells is a good model for the process which occurs in vivo.

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c) Lipid rafts isolation:

Procedures for isolating lipid rafts based on resistance to solubilization in cold Triton X-100 followed by flotation on sucrose gradients have been described by numerous laboratories (*Simons et al., 2000; Hooper et al., 1999*). Lipid rafts from the
 5 two cell lines selected above are carried out as follows:

Subconfluent cultures of N2a cells are washed in PBS and collected by centrifugation 1000 x g for 5min. Typically 3 x 15cm dishes are pooled equivalent to approximately 8×10^7 cells. The cell pellet is re-suspended in 1ml ice cold raft buffer (1% Triton in PBS, containing 10 μ M copper sulphate and a cocktail of complete
 10 protease inhibitors (Boehringer)). Cells are disrupted by seven passages through a 22G needle followed by incubation for 30 min at 4°C with gentle agitation. 2 volumes 60% sucrose in PBS is added and the lysate is transferred to a SW41 centrifuge tube. The lysate is carefully overlaid with 7 ml 35% sucrose and 1ml 15% sucrose both in PBS and centrifuged 20hr at 35,000 RPM. The lipid rafts are recovered in the top 1ml of the
 15 gradient. Membranes are concentrated by addition of 10 volumes cold PBS and centrifugation at 100,000g for 2hr. Alternatively for 2D gel electrophoresis, proteins from the lipid raft fraction are recovered by precipitation in the presence of 5 vol acetone for 2hr at -80°C. Acetone precipitates are collected by centrifugation 14000g 20min and washed twice in 70% ethanol.

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In both sensitive and resistant cells around 1-2% of protein in the total lysate is recovered in the bouyant raft fraction. As shown by Western blotting (Figure 4A) while PrP is barely detectable in the total cell extract, it is highly enriched in rafts leaving the sample layer totally depleted of PrP following centrifugation.

25 Prion infection sensitive clone #60 and the prion infection resistant clone #23 are compared by western blotting with anti-PrP (Figure 4B). Three different independent pairs of raft preparations each containing 5 μ g total raft proteins are re-probed with anti-actin antibody which confirms the uniformity of PrP protein loading (Figure 4C).

30 The results indicate that the level of PrP in the lipid raft preparations from the two cell types is indistinguishable. Moreover the distribution between non-glycosylated mono- and di-glycosylated isoforms as well as the segregation to the detergent resistant

membrane fraction shown in Figure 4A is identical suggesting that none of these factors are likely to be responsible for the differing phenotypes.

PrP cDNA was amplified by RT-PCR from both cell lines as follows:

- 5 Total RNA of N2a cells is prepared using Trizol (Gibco) and the mouse PrP cDNA is reversed transcribed with Omniscript (Qiagen) using the protocol supplied by the manufacturer. The specific primer for cDNA synthesis is 5' TCAATTGAAAGAGCTACAGGTG 3'. The prion cDNA is amplified using standard PCR conditions in the presence of primers 5' ACCAGTCCAATTTAGGAGAGCC 3' (top strand) and 5' AGACCACGAGAATGCGAAGG 3' (bottom strand). The PCR product was completely sequenced in the automated ABI3700 using the reagents and the protocol supplied by the manufacturer.

15 These data revealed that PrP mRNA is wild type in both cases and that both carry a Methionine at position 129, which in humans is the site for a frequent polymorphism.

Therefore, the expression levels, glycosylation patterns, intracellular localisation and primary sequences of PrP^C in both cell types is indistinguishable and thus that other cellular factors are responsible for the differential response to PrP^{Sc}.

20 **d) *In vitro* cyclic amplification of protein misfolding (PMCA) in lipid rafts from prion infection sensitive cells:**

Lipid rafts obtained at §c are isolated from prion infection sensitive sub-clones, #60 sub-clones, collected by centrifugation as described above and resuspended in PMCA conversion buffer at a concentration of 2-2.5 mg/ml (PBS containing final concentration of 300mM NaCl, 0.5% Triton X100, 0.05% SDS).

25 A 10% extract of RML-infected mouse brain homogenate is added directly to the rafts preparation at a dilution of 1:100 based on protein content and aliquots of the mixture are either frozen immediately, incubated for 15hr at 37 °C or subjected to 15 cycles of PMCA (5 x 0.1 second pulses of sonication followed by incubation at 37 °C for 1hr).

30 Aliquots of 20µl sample are then treated with 10 µg /ml Proteinase K for 1hr 37 °C. Lipids are removed by precipitating PK-resistant proteins with 5 vol acetone for 2hr

at -80°C . Acetone precipitates are collected by centrifugation 14000g 20min, washed twice in 70% ethanol analysed by Western blotting with 6H4 anti-PrP (Figure 5).

5 Compared to the mixture without PK treatment (lanes 1 and 5) all digested samples show a shift in molecular weight characteristic of the N-terminally truncated PK resistant form PrP₂₇₋₃₀. It should be noted that the 6H4 antibody also has low level cross reactivity with PK which migrates at 30kDa, close to the di-glycosylated form of PK-digested PrP. Analysis of the data with this in mind shows that the initial level of PK-resistant PrP derived from the diluted brain extract, which is present in the non-amplified mixtures, is barely detectable under these conditions (lanes 2).

10 A slight increase in signal is seen when the prion infection sensitive (#60) DRM is incubated at 37°C for 15 hr (lane 3 from #60), however the most dramatic increase in PK-resistant PrP is seen when this sample is subjected to 15 cycles of PMCA (lane 4 from #60). This indicates that all factors required for conversion of PrP^C to PrP^{Sc} are resident in the lipid rafts from the prion infection sensitive N2a cells. Interestingly, in
15 the parallel analysis in which the DRM from the prion infection resistant cell line #23 was used, no amplification in vitro was observed (lane 4 from #23) indicating that the capacity of the lipid rafts to convert the prion protein in vitro reflects the activity observed in the intact cells.

20 e) Effect of antibody raised against apolipoprotein B on prion replication by prion infection sensitive N2a cells:

Chronically infected sensitive cells were cultured in 24-well dishes in the presence of a goat polyclonal antibody raised against human Apo B (Chemicon) at increasing concentrations from 0 to 2mg/ml in DMEM Gibco # 41966-029, containing 1
25 x B27 supplements (Gibco #17504-044) and standard antibiotics (penicillin and streptomycin)

A parallel series of cultures was incubated in the presence of the same concentration range of total IgG from a naïve goat. The results show that concentrations of anti-hApoB antibody above 0.5mg/ml result in progressive inhibition of PrP
30 replication as revealed by quantitative dot blotting (Figure 6).

EXAMPLE 3: Proteomics analysis of lipid rafts of prion infection resistant and sensitive cells:

Since the two cell preparations are indistinguishable in terms of their PrP content a more complete protein comparison using 2D gel electrophoresis was performed to show differences in other proteins that might underline the difference in conversion activity between the two sub-clones.

2D gel preparations are prepared as follows:

Acetone precipitated proteins (see §c) are re-suspended in 20µl 1% SDS, 0.23% DTT and heated to 95°C for 5min. After the preparation is cooled to room temperature, 25µl of a solution (9M urea, 4% CHAPS, 65mM DTT, 35mM Tris base) is added. Fifteen minutes later, 85µl of a solution containing 7M urea, 2M thiourea, 4% CHAPS, 100mM DTT is further added to the mixture. After a further 15 min, non-solubilized material is removed by centrifugation at 14000 RPM during 5min and the supernatant is applied directly to a 7cm IPG strip and left to re-hydrate overnight. For IEF the voltage is progressively increased from 300V to 3.5kV and electrophoresed for a total of 20kVh. Proteins are resolved in the second dimension using single well 4-12% gradient gels (Novex) and stained using the silver express kit (Invitogen) according to the instructions supplied.

Analysis by 2D gels reveals the fraction of protein that is recovered in the lipid rafts (approximately 1-2% protein in the N2a cell lysate) as a reproducible subset of total cell proteins in which several hundred species can be visualized following silver staining (Figure 7A and B).

The 2D patterns are compared between preparations isolated from the prion infection sensitive and resistant cells. The analysis is focused on several proteins identified in the basic range of the gel, which are more abundant in DRMs from prion infection resistant cells (arrows in Figure 7C and D).

Following preparative scale electrophoresis, the two proteins indicated by arrows are excised and processed for MS sequencing. From both proteins an identical tryptic peptide is found with a monoisotopic mass of 1234.6. The N-terminal sequence of this tryptic peptide is: ENFAGEATLQR (SEQ ID NO: 3). All amino acids in the peptide are identified in the MS/MS spectrum of doubly charged precursor ion at m/z 618.30 And through its Mascot analysis.

Database searching identified this protein unambiguously as Apolipoprotein B (Apo B). Since the molecular weight of full length Apo B is in excess of 500 kDa while these two spots migrate with estimated molecular weights of 40kDa and 30kDa, we presume that the latter are fragments generated either in the cell or during sample preparation. The sequence corresponds to amino acids 3548-3558 of the human Apo B protein, which is present only in ApoB-100 and not in the truncated ApoB-48 form.

These data suggest that fragments of a molecular weight of or about 30 to 40 kDa comprising the sequence of SEQ ID NO: 3 may have a role in the prion conversion pathway.

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Claims

1. Use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof in an assay for the detection of the formation of PrP^{sc} in a sample.
5
2. Use of a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof in a screening assay for identifying compounds that modulate the conversion of PrP^c into PrP^{sc}.
10
3. Use according to claims 1 or 2 wherein the peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof forms a complex with the LDL receptor.
- 15 4. Use according to any of the preceding claims wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay.
5. Use according to any of the preceding claims wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using normal brain homogenate as a source of normal PrP^c and substrate.
20
6. Use according to claims 1 to 4 wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using lipid rafts from infection sensitive neuroblasma cell line N2a as a source of normal PrP^c and substrate.
25
7. Use of a modulator of a protein or a peptide selected from Apolipoprotein B, and fragment thereof, Apolipoprotein E and a fragment thereof for the preparation of a pharmaceutical preparation for the treatment of a prion disease.
- 30 8. Use according to claim 7 wherein the modulator antagonizes the formation of a complex between Apolipoprotein B and/or Apolipoprotein E with the LDL receptor.

9. Use according to claims 7 or 8 wherein the modulator is an antibody raised against Apolipoprotein B or a fragment thereof.
10. Use according to claims 7 or 8 wherein the modulator is an antibody raised against Apolipoprotein E or a fragment thereof.
11. Use according to any of the preceding claims wherein the peptide or the protein is Apolipoprotein B or a fragment thereof.
12. Use according to any of the preceding claims wherein the peptide of the protein contains the sequence of SEQ ID NO: 3.
13. Use according to any of the preceding claims wherein the peptide or the protein is of a molecular weight selected from 30 and 40 kDa and which sequence is selected from fragments of Apolipoprotein B taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.
14. Use according to any of the preceding claims wherein the peptide or the protein is Apolipoprotein E or a fragment thereof.
15. Use according to any one of claims 7 to 14 wherein the prion disease is bovine spongiform encephalopathy (BSE).
16. Use according to any one of claims 7 to 14 wherein the prion disease is a Creutzfeld-Jacob Disease (CJD).
17. A method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) obtaining a sample from the subject; (ii) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof; (iii) contacting the mixture obtained in

step (ii) with PrP^C or PrP^C containing mixtures; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample.

- 5 18. A method of determining a marker that predisposes a subject to a prion disease, comprising (i) obtaining a sample from the subject; (ii) measuring a level of a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof in said sample; and (iii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease.
- 10 19. A method for the detection of PrP^{Sc} within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof; (ii) contacting sample obtained in (i) with PrP^C or PrP^C containing mixtures; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.
- 15 20. A method according to any one of claims 17 to 19 wherein the prion disease is bovine spongiform encephalopathy (BSE).
- 20 21. A method according to any one of claims 17 to 19 wherein the prion disease is a Creutzfeld-Jacob disease.
- 25 22. A method for identifying a compound which modulates the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof (a) in the presence of said modulatory compound and (b) in the absence of said compound; (ii) contacting the mixtures obtained in step (i) a and (i) b with PrP^C or PrP^C containing mixtures; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said modulatory compound and (b) in the absence of said modulatory compound.
- 30 23. An assay for the detection of PrP^{Sc} in a sample within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from

Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof; (ii) contacting the mixture obtained in step (i) with PrP^C or PrP^C containing mixtures; (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

5 24. A screening assay for identifying a compound which modulates the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof (a) in the presence of said modulatory compound and (b) in the absence of said modulatory compound; (ii) contacting the mixtures obtained in
10 step (i) a and (i) b with PrP^C or PrP^C containing mixtures; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said modulatory compound.

15 25. A diagnostic kit for use in the assay according to claim 23, comprising a probe for receiving a sample and a peptide or a protein selected from Apolipoprotein B, a fragment thereof, Apolipoprotein E and a fragment thereof.

20 26. An apparatus for use in the method of any one of claims 17 to 22 or the assay of any one claims 23 to 24.

Abstract

The use of apolipoprotein B or apolipoprotein E or fragments thereof is provided for diagnostic, detection, prognostic and therapeutic applications in prion diseases. More specifically, the invention provides the use of apolipoprotein B or apolipoprotein E or
5 fragments thereof for modulating or identifying modulators of the prion protein replication which are implicated in the pathogenesis of transmissible spongiform encephalopathies and other prion diseases.

Figure 1

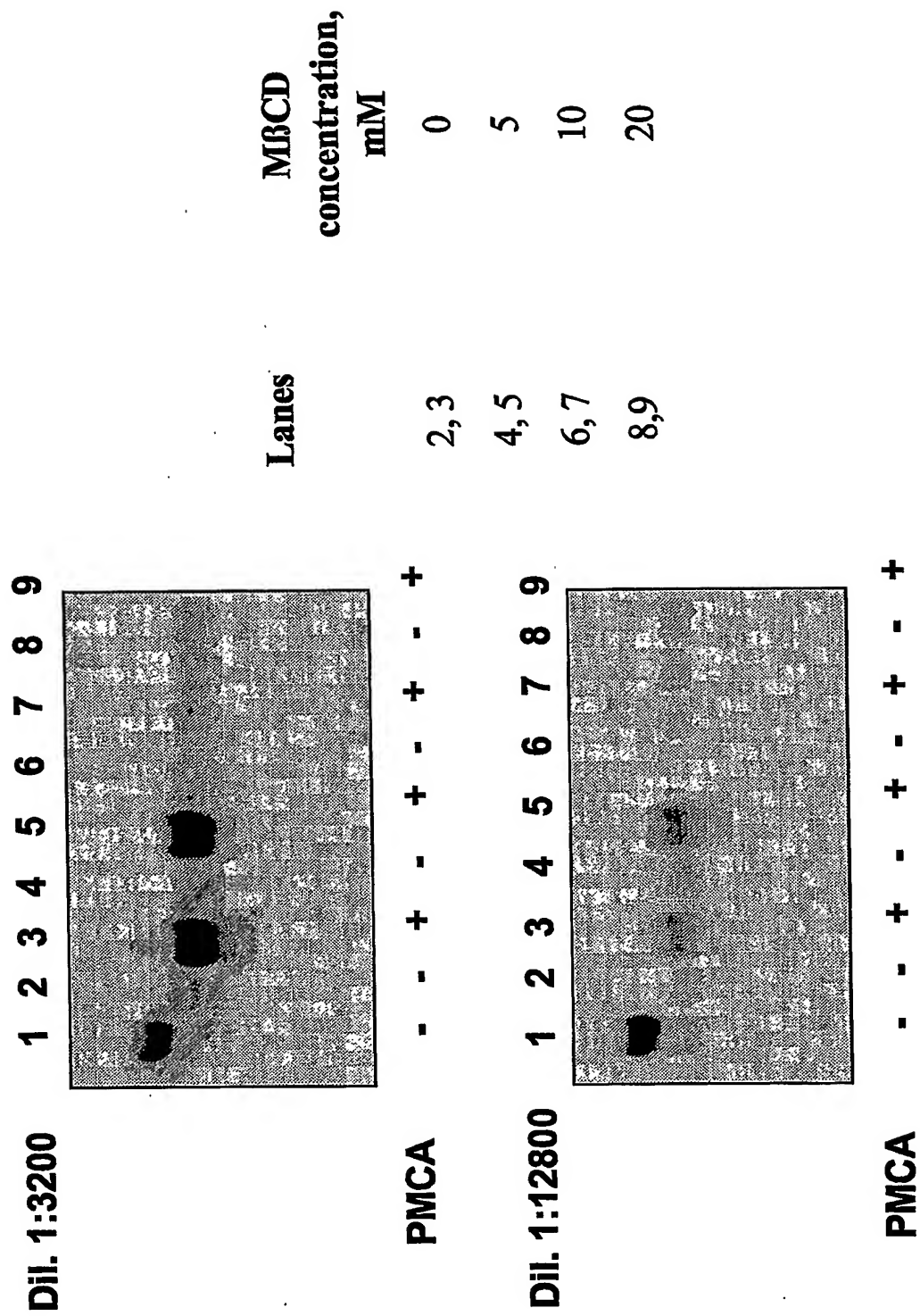


Figure 2A

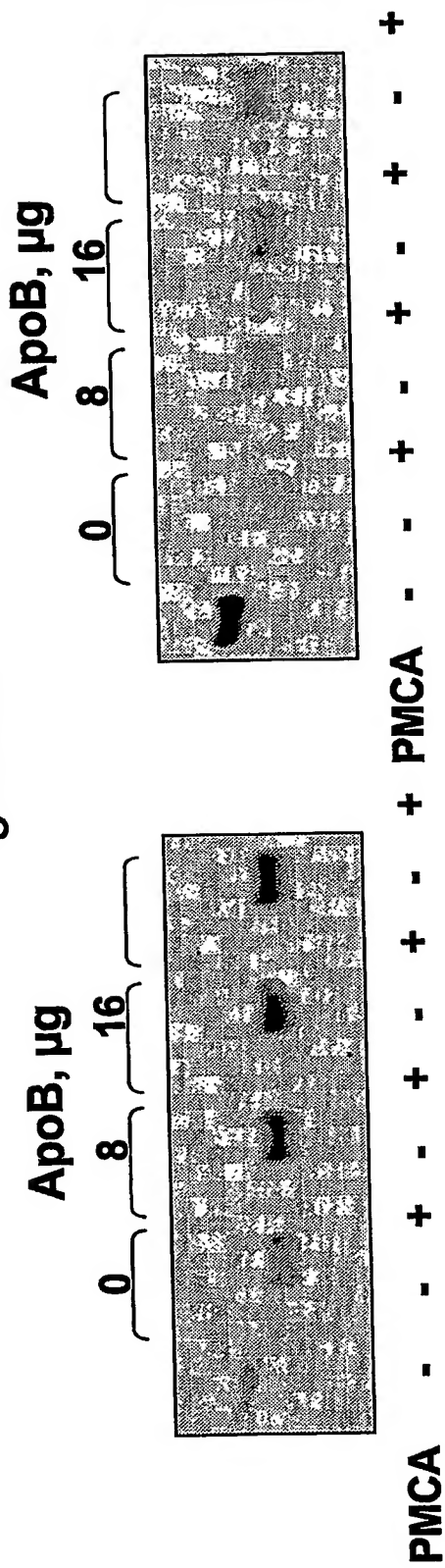


Figure 2B

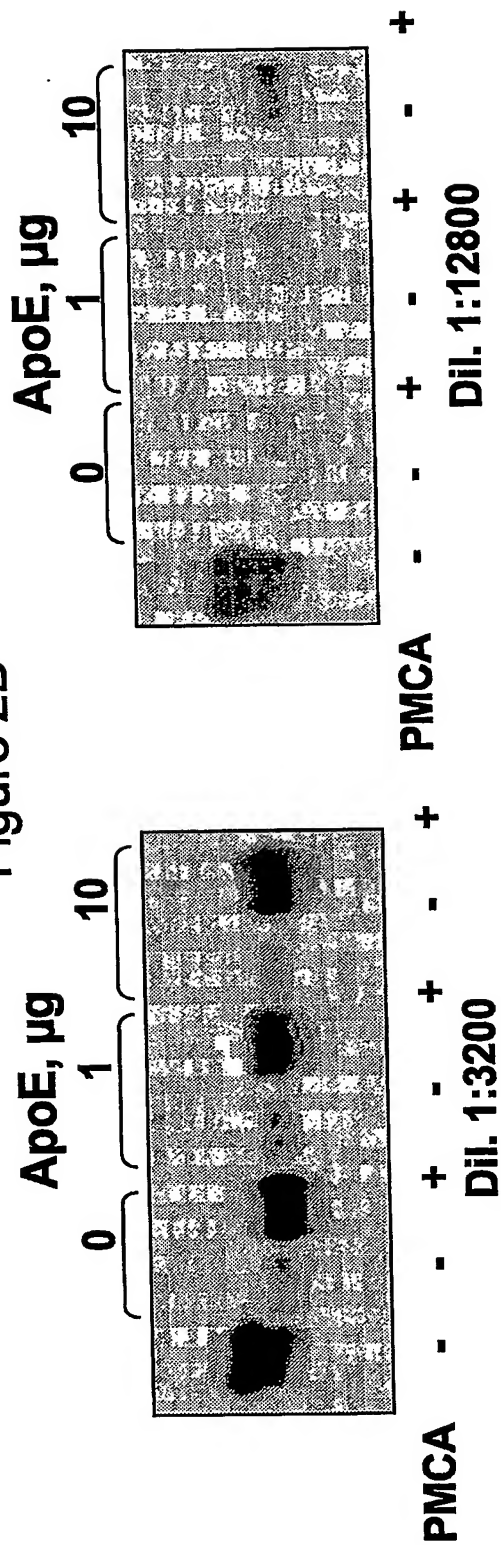


Figure 2C

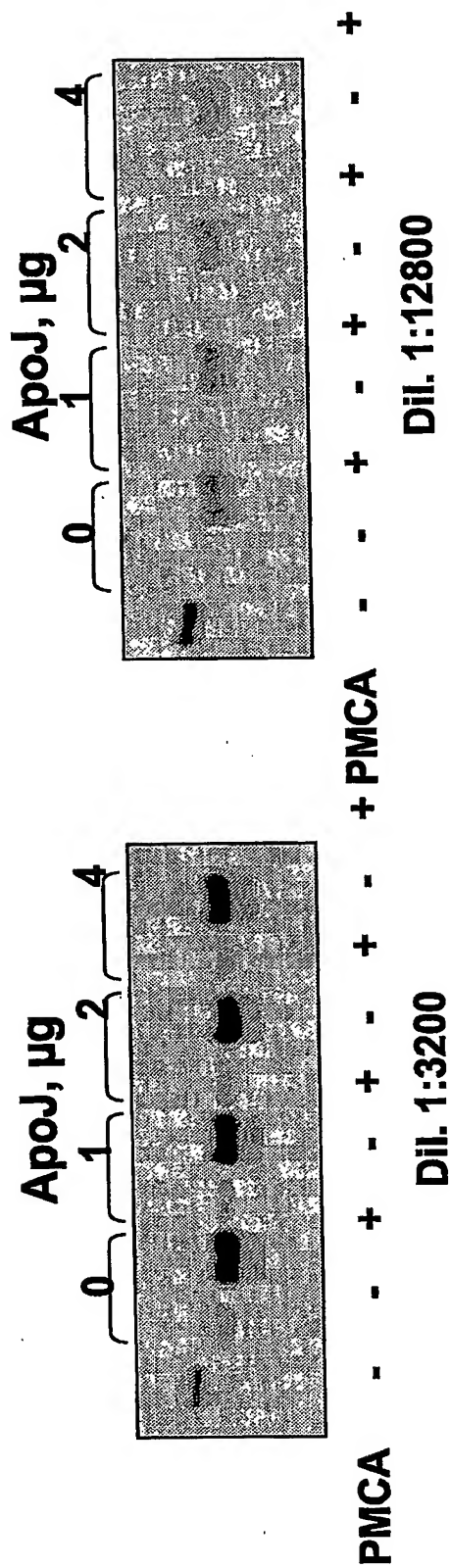


Figure 3

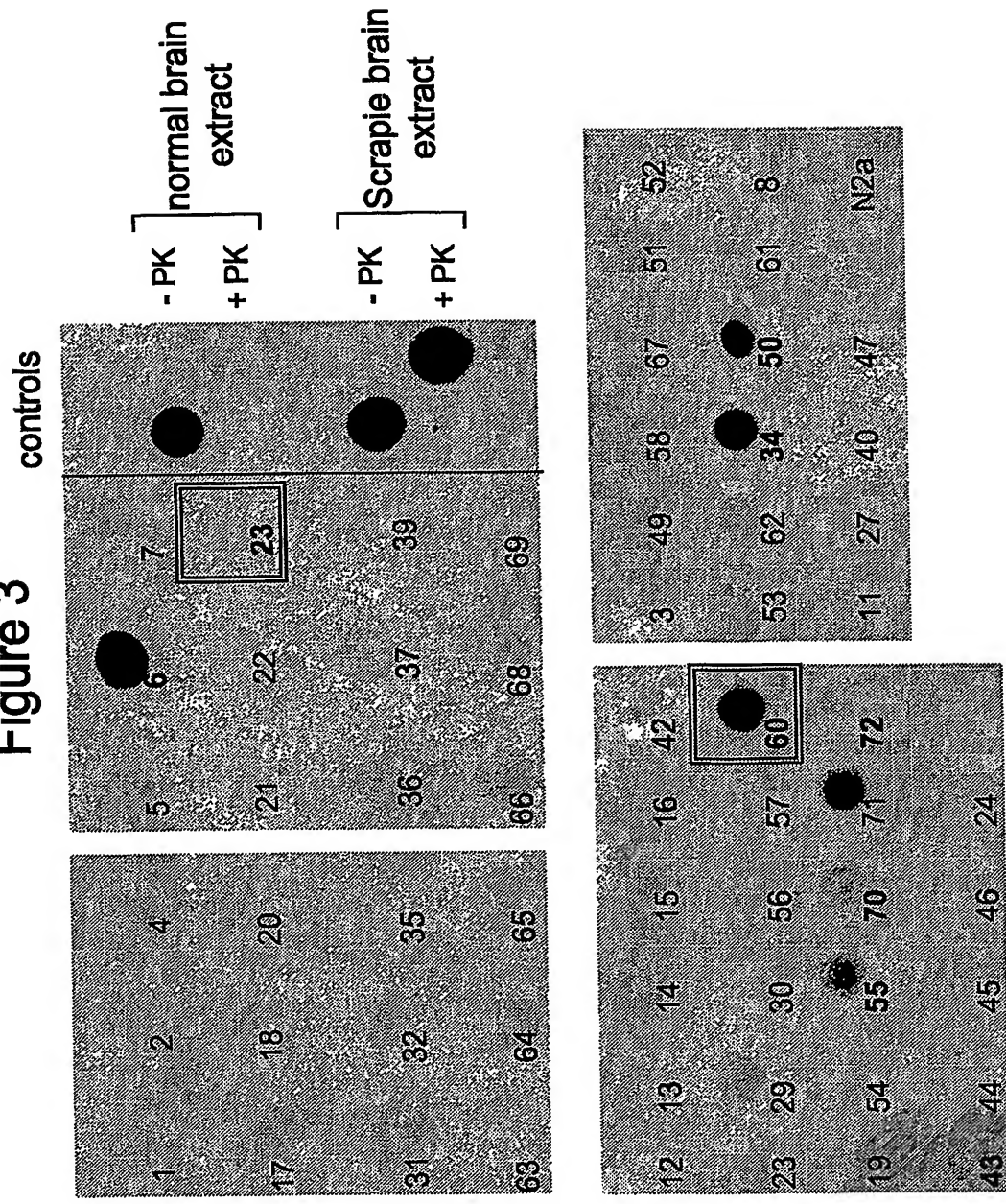


Figure 4

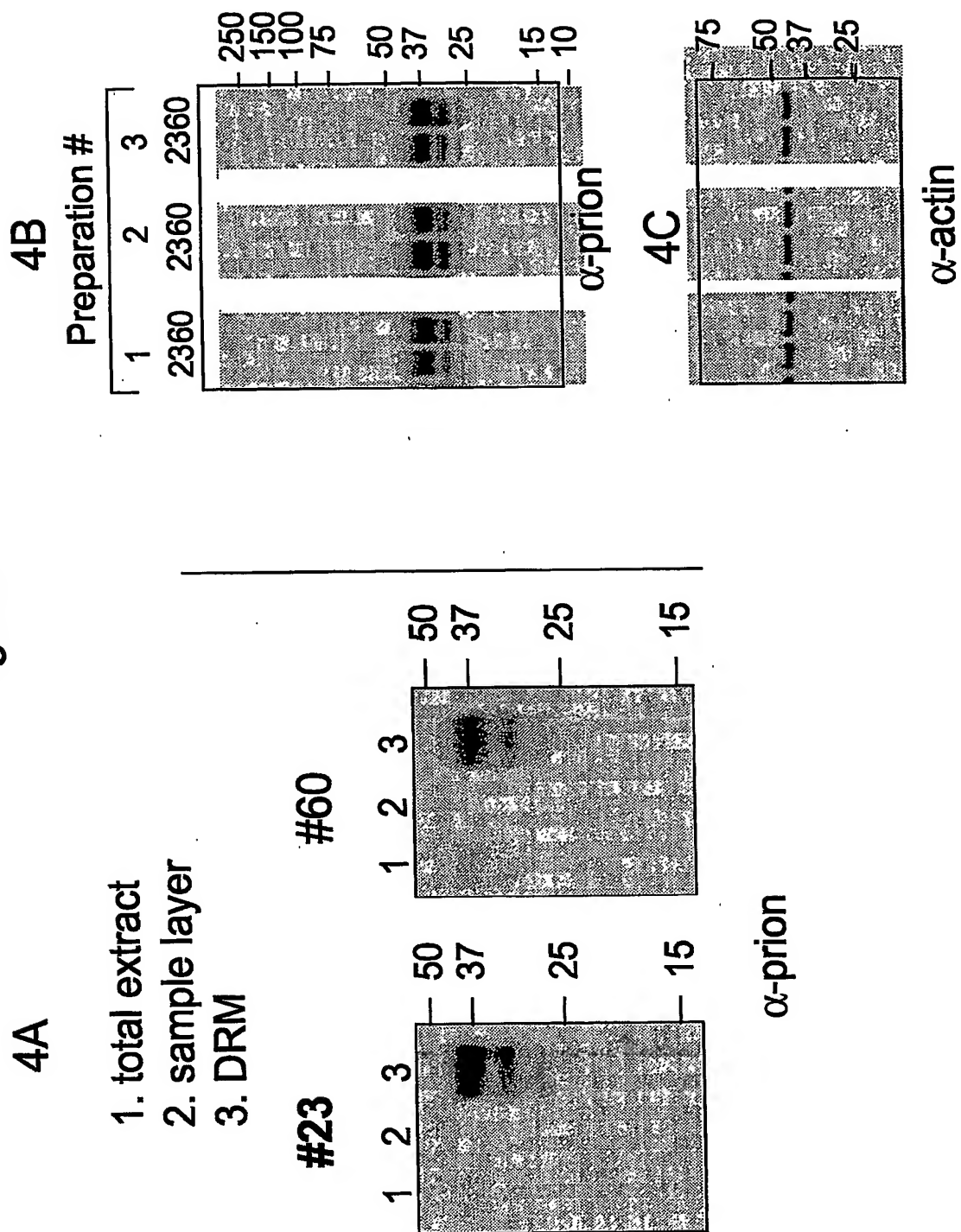


Figure 5

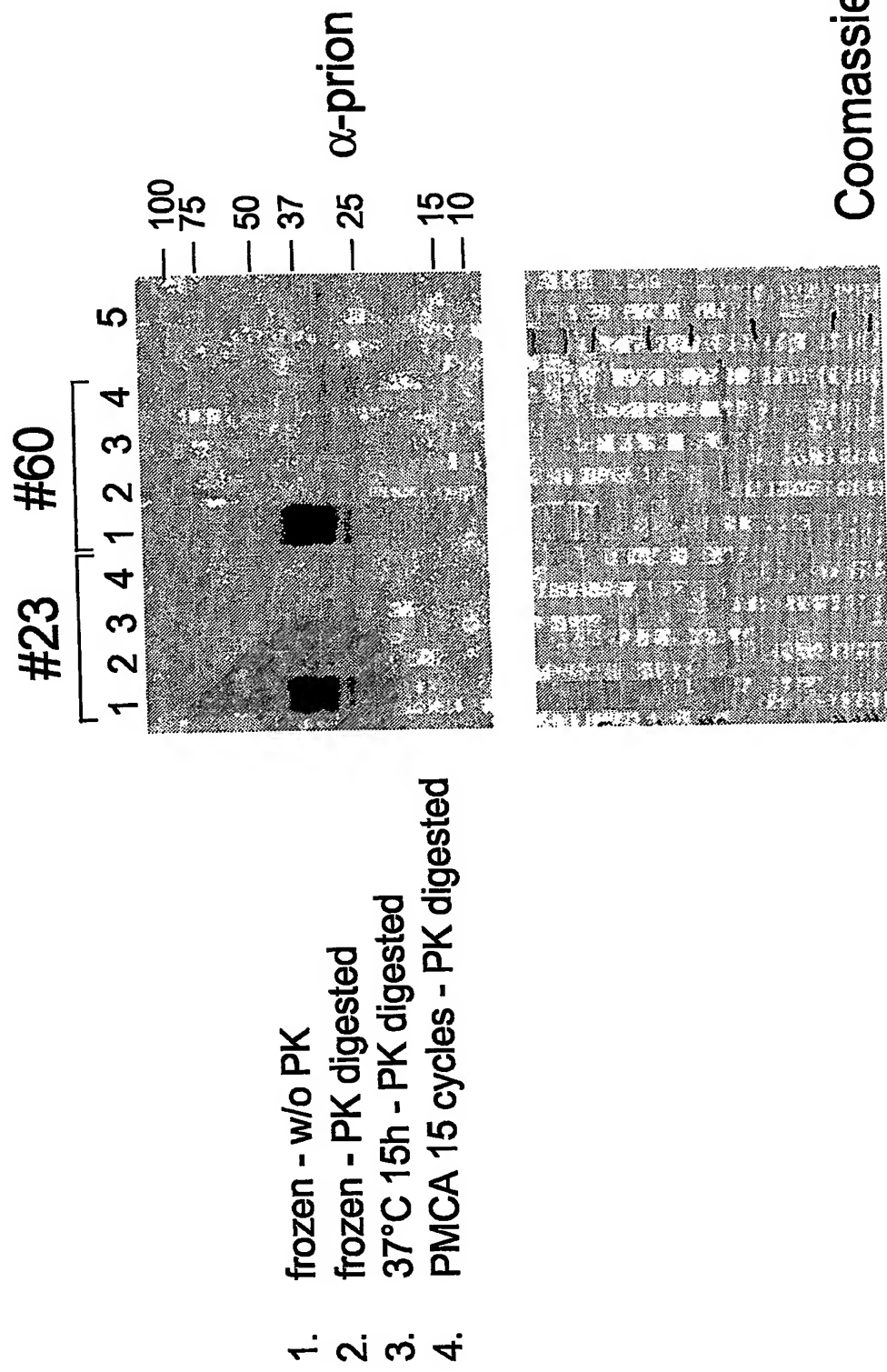


Figure 6

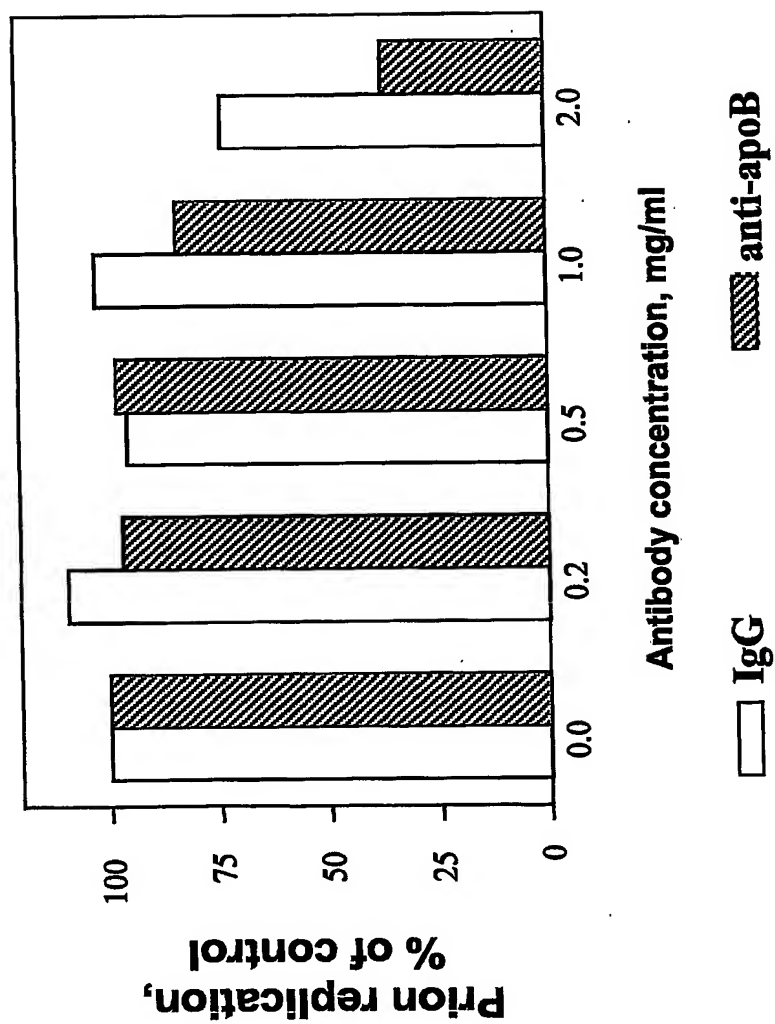
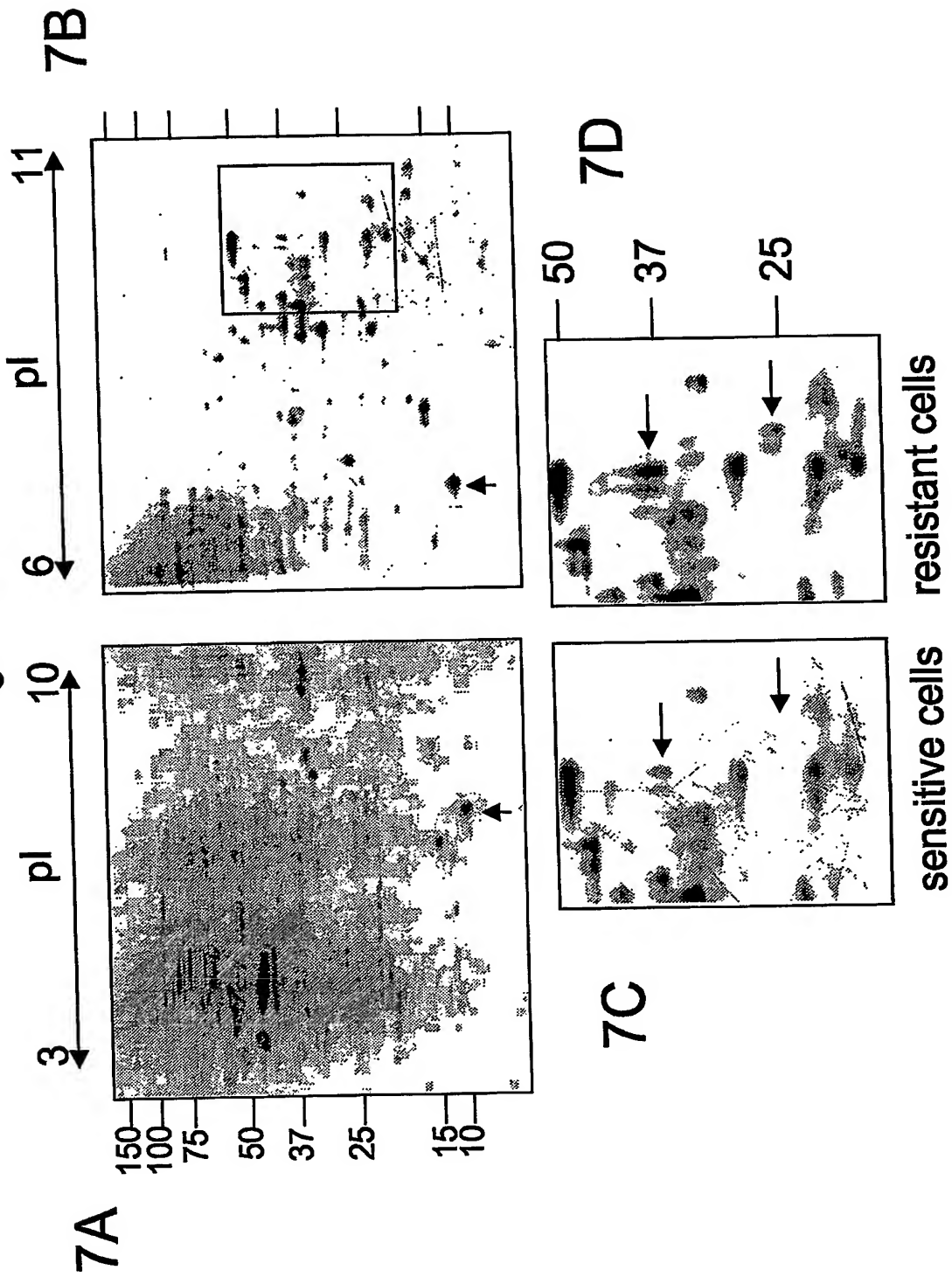


Figure 7



SEQUENCE LISTING

<110> APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.

<120> USE OF PRION CONVERSION MODULATING AGENTS

<130> EP/845

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<170> PatentIn version 3.1

<210> 1

<211> 4563

<212> PRT

<213> Homo sapiens

<400> 1

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 50 55 60

Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
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Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
 85 90 95

Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
 100 105 110

Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
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Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg Gly Ile
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Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Ser Thr Ser Pro Pro Lys 305 310 315 320		
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 915 920 925

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Phe Leu 2420	Asp Met Leu Ile 2425	Lys Lys Leu Lys Ser Phe 2430	Asp Tyr His
Gln Phe 2435	Val Asp Glu Thr 2440	Asn Asp Lys Ile Arg Glu 2445	Val Thr Gln
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Ser	Ile	Gln	Asp	Trp	Ala	Lys	Arg	Met	Lys	Ala	Leu	Val	Glu	Gln
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2600						2605					2610			
Thr	Pro	Asp	Phe	Ile	Val	Pro	Leu	Thr	Asp	Leu	Arg	Ile	Pro	Ser
2615						2620					2625			
Val	Gln	Ile	Asn	Phe	Lys	Asp	Leu	Lys	Asn	Ile	Lys	Ile	Pro	Ser
2630						2635					2640			
Arg	Phe	Ser	Thr	Pro	Glu	Phe	Thr	Ile	Leu	Asn	Thr	Phe	His	Ile
2645						2650					2655			
Pro	Ser	Phe	Thr	Ile	Asp	Phe	Val	Glu	Met	Lys	Val	Lys	Ile	Ile
2660						2665					2670			
Arg	Thr	Ile	Asp	Gln	Met	Gln	Asn	Ser	Glu	Leu	Gln	Trp	Pro	Val
2675						2680					2685			
Pro	Asp	Ile	Tyr	Leu	Arg	Asp	Leu	Lys	Val	Glu	Asp	Ile	Pro	Leu
2690						2695					2700			
Ala	Arg	Ile	Thr	Leu	Pro	Asp	Phe	Arg	Leu	Pro	Glu	Ile	Ala	Ile
2705						2710					2715			
Pro	Glu	Phe	Ile	Ile	Pro	Thr	Leu	Asn	Leu	Asn	Asp	Phe	Gln	Val
2720						2725					2730			

Pro	Asp	Leu	His	Ile	Pro	Glu	Phe	Gln	Leu	Pro	His	Ile	Ser	His
2735						2740					2745			
Thr	Ile	Glu	Val	Pro	Thr	Phe	Gly	Lys	Leu	Tyr	Ser	Ile	Leu	Lys
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Ile	Gln	Ser	Pro	Leu	Phe	Thr	Leu	Asp	Ala	Asn	Ala	Asp	Ile	Gly
2765						2770					2775			
Asn	Gly	Thr	Thr	Ser	Ala	Asn	Glu	Ala	Gly	Ile	Ala	Ala	Ser	Ile
2780						2785					2790			
Thr	Ala	Lys	Gly	Glu	Ser	Lys	Leu	Glu	Val	Leu	Asn	Phe	Asp	Phe
2795						2800					2805			
Gln	Ala	Asn	Ala	Gln	Leu	Ser	Asn	Pro	Lys	Ile	Asn	Pro	Leu	Ala
2810						2815					2820			
Leu	Lys	Glu	Ser	Val	Lys	Phe	Ser	Ser	Lys	Tyr	Leu	Arg	Thr	Glu
2825						2830					2835			
His	Gly	Ser	Glu	Met	Leu	Phe	Phe	Gly	Asn	Ala	Ile	Glu	Gly	Lys
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Asp	Phe	Ser	Ser	Gln	Ala	Asp	Leu	Arg	Asn	Glu	Ile	Lys	Thr	Leu
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Leu	Lys	Ala	Gly	His	Ile	Ala	Trp	Thr	Ser	Ser	Gly	Lys	Gly	Ser
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Trp	Lys	Trp	Ala	Cys	Pro	Arg	Phe	Ser	Asp	Glu	Gly	Thr	His	Glu
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Leu Val Tyr Glu Ser Gly Ser	Leu Asn Phe Ser Lys	Leu Glu Ile		
2975	2980	2985		
Gln Ser	Gln Val Asp Ser Gln	His Val Gly His Ser	Val Leu Thr	
2990	2995	3000		
Ala Lys	Gly Met Ala Leu Phe	Gly Glu Gly Lys Ala	Glu Phe Thr	
3005	3010	3015		
Gly Arg	His Asp Ala His Leu	Asn Gly Lys Val Ile	Gly Thr Leu	
3020	3025	3030		
Lys Asn	Ser Leu Phe Phe Ser	Ala Gln Pro Phe Glu	Ile Thr Ala	
3035	3040	3045		
Ser Thr	Asn Asn Glu Gly Asn	Leu Lys Val Arg Phe	Pro Leu Arg	
3050	3055	3060		
Leu Thr	Gly Lys Ile Asp Phe	Leu Asn Asn Tyr Ala	Leu Phe Leu	
3065	3070	3075		
Ser Pro	Ser Ala Gln Gln Ala	Ser Trp Gln Val Ser	Ala Arg Phe	
3080	3085	3090		
Asn Gln	Tyr Lys Tyr Asn Gln	Asn Phe Ser Ala Gly	Asn Asn Glu	
3095	3100	3105		
Asn Ile	Met Glu Ala His Val	Gly Ile Asn Gly Glu	Ala Asn Leu	
3110	3115	3120		
Asp Phe	Leu Asn Ile Pro Leu	Thr Ile Pro Glu Met	Arg Leu Pro	
3125	3130	3135		
Tyr Thr	Ile Ile Thr Thr Pro	Pro Leu Lys Asp Phe	Ser Leu Trp	
3140	3145	3150		
Glu Lys	Thr Gly Leu Lys Glu	Phe Leu Lys Thr Thr	Lys Gln Ser	
3155	3160	3165		
Phe Asp	Leu Ser Val Lys Ala	Gln Tyr Lys Lys Asn	Lys His Arg	
3170	3175	3180		
His Ser	Ile Thr Asn Pro Leu	Ala Val Leu Cys Glu	Phe Ile Ser	
3185	3190	3195		

Gln Ser	Ile Lys Ser Phe Asp	Arg His Phe Glu Lys	Asn Arg Asn
3200	3205	3210	
Asn Ala	Leu Asp Phe Val Thr	Lys Ser Tyr Asn Glu	Thr Lys Ile
3215	3220	3225	
Lys Phe	Asp Lys Tyr Lys Ala	Glu Lys Ser His Asp	Glu Leu Pro
3230	3235	3240	
Arg Thr	Phe Gln Ile Pro Gly	Tyr Thr Val Pro Val	Val Asn Val
3245	3250	3255	
Glu Val	Ser Pro Phe Thr Ile	Glu Met Ser Ala Phe	Gly Tyr Val
3260	3265	3270	
Phe Pro	Lys Ala Val Ser Met	Pro Ser Phe Ser Ile	Leu Gly Ser
3275	3280	3285	
Asp Val	Arg Val Pro Ser Tyr	Thr Leu Ile Leu Pro	Ser Leu Glu
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Leu Pro	Val Leu His Val Pro	Arg Asn Leu Lys Leu	Ser Leu Pro
3305	3310	3315	
His Phe	Lys Glu Leu Cys Thr	Ile Ser His Ile Phe	Ile Pro Ala
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Met Gly	Asn Ile Thr Tyr Asp	Phe Ser Phe Lys Ser	Ser Val Ile
3335	3340	3345	
Thr Leu	Asn Thr Asn Ala Glu	Leu Phe Asn Gln Ser	Asp Ile Val
3350	3355	3360	
Ala His	Leu Leu Ser Ser Ser	Ser Ser Val Ile Asp	Ala Leu Gln
3365	3370	3375	
Tyr Lys	Leu Glu Gly Thr Thr	Arg Leu Thr Arg Lys	Arg Gly Leu
3380	3385	3390	
Lys Leu	Ala Thr Ala Leu Ser	Leu Ser Asn Lys Phe	Val Glu Gly
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Ser His	Asn Ser Thr Val Ser	Leu Thr Thr Lys Asn	Met Glu Val
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Ser Val Ala Lys Thr Thr Lys Ala Glu Ile Pro Ile Leu Arg Met
 3425 3430 3435

Asn Phe Lys Gln Glu Leu Asn Gly Asn Thr Lys Ser Lys Pro Thr
 3440 3445 3450

Val Ser Ser Ser Met Glu Phe Lys Tyr Asp Phe Asn Ser Ser Met
 3455 3460 3465

Leu Tyr Ser Thr Ala Lys Gly Ala Val Asp His Lys Leu Ser Leu
 3470 3475 3480

Glu Ser Leu Thr Ser Tyr Phe Ser Ile Glu Ser Ser Thr Lys Gly
 3485 3490 3495

Asp Val Lys Gly Ser Val Leu Ser Arg Glu Tyr Ser Gly Thr Ile
 3500 3505 3510

Ala Ser Glu Ala Asn Thr Tyr Leu Asn Ser Lys Ser Thr Arg Ser
 3515 3520 3525

Ser Val Lys Leu Gln Gly Thr Ser Lys Ile Asp Asp Ile Trp Asn
 3530 3535 3540

Leu Glu Val Lys Glu Asn Phe Ala Gly Glu Ala Thr Leu Gln Arg
 3545 3550 3555

Ile Tyr Ser Leu Trp Glu His Ser Thr Lys Asn His Leu Gln Leu
 3560 3565 3570

Glu Gly Leu Phe Phe Thr Asn Gly Glu His Thr Ser Lys Ala Thr
 3575 3580 3585

Leu Glu Leu Ser Pro Trp Gln Met Ser Ala Leu Val Gln Val His
 3590 3595 3600

Ala Ser Gln Pro Ser Ser Phe His Asp Phe Pro Asp Leu Gly Gln
 3605 3610 3615

Glu Val Ala Leu Asn Ala Asn Thr Lys Asn Gln Lys Ile Arg Trp
 3620 3625 3630

Lys Asn Glu Val Arg Ile His Ser Gly Ser Phe Gln Ser Gln Val
 3635 3640 3645

Glu Leu	Ser Asn Asp Gln Glu	Lys Ala His Leu Asp	Ile Ala Gly
3650	3655	3660	
Ser Leu	Glu Gly His Leu Arg	Phe Leu Lys Asn Ile	Ile Leu Pro
3665	3670	3675	
Val Tyr	Asp Lys Ser Leu Trp	Asp Phe Leu Lys Leu	Asp Val Thr
3680	3685	3690	
Thr Ser	Ile Gly Arg Arg Gln	His Leu Arg Val Ser	Thr Ala Phe
3695	3700	3705	
Val Tyr	Thr Lys Asn Pro Asn	Gly Tyr Ser Phe Ser	Ile Pro Val
3710	3715	3720	
Lys Val	Leu Ala Asp Lys Phe	Ile Thr Pro Gly Leu	Lys Leu Asn
3725	3730	3735	
Asp Leu	Asn Ser Val Leu Val	Met Pro Thr Phe His	Val Pro Phe
3740	3745	3750	
Thr Asp	Leu Gln Val Pro Ser	Cys Lys Leu Asp Phe	Arg Glu Ile
3755	3760	3765	
Gln Ile	Tyr Lys Lys Leu Arg	Thr Ser Ser Phe Ala	Leu Asn Leu
3770	3775	3780	
Pro Thr	Leu Pro Glu Val Lys	Phe Pro Glu Val Asp	Val Leu Thr
3785	3790	3795	
Lys Tyr	Ser Gln Pro Glu Asp	Ser Leu Ile Pro Phe	Phe Glu Ile
3800	3805	3810	
Thr Val	Pro Glu Ser Gln Leu	Thr Val Ser Gln Phe	Thr Leu Pro
3815	3820	3825	
Lys Ser	Val Ser Asp Gly Ile	Ala Ala Leu Asp Leu	Asn Ala Val
3830	3835	3840	
Ala Asn	Lys Ile Ala Asp Phe	Glu Leu Pro Thr Ile	Ile Val Pro
3845	3850	3855	
Glu Gln	Thr Ile Glu Ile Pro	Ser Ile Lys Phe Ser	Val Pro Ala
3860	3865	3870	
Gly Ile	Val Ile Pro Ser Phe	Gln Ala Leu Thr Ala	Arg Phe Glu

3875	3880	3885
Val Asp 3890	Ser Pro Val Tyr Asn 3895	Ala Thr Trp Ser Ala 3900
Asn Lys 3905	Ala Asp Tyr Val Glu 3910	Thr Val Leu Asp Ser 3915
Ser Thr 3920	Val Gln Phe Leu Glu 3925	Tyr Glu Leu Asn Val 3930
His Lys 3935	Ile Glu Asp Gly Thr 3940	Leu Ala Ser Lys Thr 39 45
Leu Ala 3950	His Arg Asp Phe Ser 3955	Ala Glu Tyr Glu Glu 3960
Phe Glu 3965	Gly Leu Gln Glu Trp 3970	Glu Gly Lys Ala His 3975
Lys Ser 3980	Pro Ala Phe Thr Asp 3985	Leu His Leu Arg Tyr 3990
Lys Lys 3995	Gly Ile Ser Thr Ser 4000	Ala Ala Ser Pro Ala 4005
Val Gly 4010	Met Asp Met Asp Glu 4015	Asp Asp Asp Phe Ser 4020
Phe Tyr 4025	Tyr Ser Pro Gln Ser 4030	Ser Pro Asp Lys Lys 4035
Phe Lys 4040	Thr Glu Leu Arg Val 4045	Arg Glu Ser Asp Glu 4050
Ile Lys 4055	Val Asn Trp Glu Glu 4060	Glu Ala Ala Ser Gly 4065
Ser Leu 4070	Lys Asp Asn Val Pro 4075	Lys Ala Thr Gly Val 4080
Tyr Val 4085	Asn Lys Tyr His Trp 409 0	Glu His Thr Gly Leu 4095
Glu Val 4100	Ser Ser Lys Leu Arg 4105	Arg Asn Leu Gln Asn 4110
		Asn Ala Glu

Trp Val Tyr Gln Gly Ala Ile Arg Gln Ile Asp Asp Ile Asp Val
 4115 4120 4125

Arg Phe Gln Lys Ala Ala Ser Gly Thr Thr Gly Thr Tyr Gln Glu
 4130 4135 4140

Trp Lys Asp Lys Ala Gln Asn Leu Tyr Gln Glu Leu Leu Thr Gln
 4145 4150 4155

Glu Gly Gln Ala Ser Phe Gln Gly Leu Lys Asp Asn Val Phe Asp
 4160 4165 4170

Gly Leu Val Arg Val Thr Gln Lys Phe His Met Lys Val Lys His
 4175 4180 4185

Leu Ile Asp Ser Leu Ile Asp Phe Leu Asn Phe Pro Arg Phe Gln
 4190 4195 4200

Phe Pro Gly Lys Pro Gly Ile Tyr Thr Arg Glu Glu Leu Cys Thr
 4205 4210 4215

Met Phe Ile Arg Glu Val Gly Thr Val Leu Ser Gln Val Tyr Ser
 4220 4225 4230

Lys Val His Asn Gly Ser Glu Ile Leu Phe Ser Tyr Phe Gln Asp
 4235 4240 4245

Leu Val Ile Thr Leu Pro Phe Glu Leu Arg Lys His Lys Leu Ile
 4250 4255 4260

Asp Val Ile Ser Met Tyr Arg Glu Leu Leu Lys Asp Leu Ser Lys
 4265 4270 4275

Glu Ala Gln Glu Val Phe Lys Ala Ile Gln Ser Leu Lys Thr Thr
 4280 4285 4290

Glu Val Leu Arg Asn Leu Gln Asp Leu Leu Gln Phe Ile Phe Gln
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Leu Ile Glu Asp Asn Ile Lys Gln Leu Lys Glu Met Lys Phe Thr
 4310 4315 4320

Tyr Leu Ile Asn Tyr Ile Gln Asp Glu Ile Asn Thr Ile Phe A sn
 4325 4330 4335

Asp Tyr Ile Pro Tyr Val Phe Lys Leu Leu Lys Glu Asn Leu Cys
 4340 4345 4350

Leu Asn Leu His Lys Phe Asn Glu Phe Ile Gln Asn Glu Leu Gln
 4355 4360 4365

Glu Ala Ser Gln Glu Leu Gln Gln Ile His Gln Tyr Ile Met Ala
 4370 4375 4380

Leu Arg Glu Glu Tyr Phe Asp Pro Ser Ile Val Gly Trp Thr Val
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Lys Tyr Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn
 4400 4405 4410

Leu Leu Val Ala Leu Lys Asp Phe His Ser Glu Tyr Ile Val Ser
 4415 4420 4425

Ala Ser Asn Phe Thr Ser Gln Leu Ser Ser Gln Val Glu Gln Phe
 4430 4435 4440

Leu His Arg Asn Ile Gln Glu Tyr Leu Ser Ile Leu Thr Asp Pro
 4445 4450 4455

Asp Gly Lys Gly Lys Glu Lys Ile Ala Glu Leu Ser Ala Thr Ala
 4460 4465 4470

Gln Glu Ile Ile Lys Ser Gln Ala Ile Ala Thr Lys Lys Ile Ile
 4475 4480 4485

Ser Asp Tyr His Gln Gln Phe Arg Tyr Lys Leu Gln Asp Phe Ser
 4490 4495 4500

Asp Gln Leu Ser Asp Tyr Tyr Glu Lys Phe Ile Ala Glu Ser Lys
 4505 4510 4515

Arg Leu Ile Asp Leu Ser Ile Gln Asn Tyr His Thr Phe Leu Ile
 4520 4525 4530

Tyr Ile Thr Glu Leu Leu Lys Lys Leu Gln Ser Thr Thr Val Met
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Asn Pro Tyr Met Lys Leu Ala Pro Gly Glu Leu Thr Ile Ile Leu
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			20					25					30		
Arg	Gln	Gln	Thr	Glu	Trp	Gln	Ser	Gly	Gln	Arg	Trp	Glu	Leu	Ala	Leu
			35				40					45			
Gly	Arg	Phe	Trp	Asp	Tyr	Leu	Arg	Trp	Val	Gln	Thr	Leu	Ser	Glu	Gln
	50					55					60				
Val	Gln	Glu	Glu	Leu	Leu	Ser	Ser	Gln	Val	Thr	Gln	Glu	Leu	Arg	Ala
65					70					75				80	
Leu	Met	Asp	Glu	Thr	Met	Lys	Glu	Leu	Lys	Ala	Tyr	Lys	Ser	Glu	Leu
				85					90					95	
Glu	Glu	Gln	Leu	Thr	Pro	Val	Ala	Glu	Glu	Thr	Arg	Ala	Arg	Leu	Ser
			100					105					110		
Lys	Glu	Leu	Gln	Ala	Ala	Gln	Ala	Arg	Leu	Gly	Ala	Asp	Met	Glu	Asp
		115					120					125			
Val	Cys	Gly	Arg	Leu	Val	Gln	Tyr	Arg	Gly	Glu	Val	Gln	Ala	Met	Leu
	130					135					140				
Gly	Gln	Ser	Thr	Glu	Glu	Leu	Arg	Val	Arg	Leu	Ala	Ser	His	Leu	Arg
145					150					155				160	
Lys	Leu	Arg	Lys	Arg	Leu	Leu	Arg	Asp	Ala	Asp	Asp	Leu	Gln	Lys	Arg
				165					170					175	
Leu	Ala	Val	Tyr	Gln	Ala	Gly	Ala	Arg	Glu	Gly	Ala	Glu	Arg	Gly	Leu
			180					185					190		
Ser	Ala	Ile	Arg	Glu	Arg	Leu	Gly	Pro	Leu	Val	Glu	Gln	Gly	Arg	Val
		195					200					205			
Arg	Ala	Ala	Thr	Val	Gly	Ser	Leu	Ala	Gly	Gln	Pro	Leu	Gln	Glu	Arg
	210					215					220				

Ala Gln Ala Trp Gly Glu Arg Leu Arg Ala Arg Met Glu Glu Met Gly
 225 230 235 240

Ser Arg Thr Arg Asp Arg Leu Asp Glu Val Lys Glu Gln Val Ala Glu
 245 250 255

Val Arg Ala Lys Leu Glu Glu Gln Ala Gln Gln Ile Arg Leu Gln Ala
 260 265 270

Glu Ala Phe Gln Ala Arg Leu Lys Ser Trp Phe Glu Pro Leu Val Glu
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Asp Met Gln Arg Gln Trp Ala Gly Leu Val Glu Lys Val Gln Ala Ala
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Val Gly Thr Ser Ala Ala Pro Val Pro Ser Asp Asn His
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<400> 3

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